

Award Number: W81XWH-12-1-0620

TITLE: Mapping the vasculome for biomarkers in TBI

PRINCIPAL INVESTIGATOR: Eng H. Lo

CONTRACTING ORGANIZATION: Massachusetts General Hospital, Boston, MA 02114-2554

REPORT DATE: October 2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

<p>Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</p>				
1. REPORT DATE October 2013	2. REPORT TYPE Annual	3. DATES COVERED 30 September 2012-29 September 2013		
4. TITLE AND SUBTITLE Mapping the vasculome for biomarkers in TBI		5a. CONTRACT NUMBER		
		5b. GRANT NUMBER W81XWH-12-1-0620		
		5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Eng H. Lo E-Mail: Lo@helix.mgh.harvard.edu		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Massachusetts General Hospital, Boston, MA 02114-2554		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT Mild-to-moderate TBI is the signature injury of US military personnel. Thus, there is an urgent need to find biomarkers for mild-to-moderate TBI. In this proposal, our plan was to develop a "vasculome" database for TBI brain. By mapping the vasculome (<i>i.e. entire gene expression profile in blood vessels</i>) using mouse models of TBI, we will generate a comprehensive database that can be mined by the research community for potentially novel biomarkers of TBI. In this annual report, we describe our progress for the first year of our research, <i>i.e.</i> completion of all <i>in vivo</i> TBI mouse models, measurement of neurological deficits in all TBI mice, and the isolation of TBI microvessels and mRNA extraction.				
15. SUBJECT TERMS TBI, mechanisms, biomarkers, endothelial, microvessels, vascular				
16. SECURITY CLASSIFICATION OF: a. REPORT U		17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
b. ABSTRACT U				19b. TELEPHONE NUMBER (include area code)
c. THIS PAGE U				

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusion.....	9
References.....	NA
Appendices.....	NA

INTRODUCTION

Mild-to-moderate TBI is the signature injury of US military personnel. Thus, there is an urgent need to find biomarkers for mild-to-moderate TBI. In this proposal, our plan was to develop a “vasculome” database for TBI brain. By mapping the ***vasculome (i.e. entire gene expression profile in blood vessels)*** using mouse models of TBI, we will generate a comprehensive database that can be mined by the research community for potentially novel biomarkers of TBI. Our new approach is based on the fact that, in terms of surface area, the blood vessel endothelium is overwhelmingly the largest organ that secretes proteins into blood. Because the endothelium can survey the entire brain, they act as cellular integrators and sensors of brain dysfunction, releasing measurable biomarkers into the circulation. Thus, we propose that mapping the vasculome of stressed blood vessels in brain will generate a database for mining entirely novel TBI biomarkers in military personnel. We will build our vasculome database using mouse models of TBI - controlled cortical impact or closed head concussive injury. We cut away the core, i.e. directly damaged brain tissue. Then we extract blood vessels from non-directly-damaged ipsilateral hemisphere and contralateral hemisphere. Controls are collected from normal sham-operated brains. Samples are obtained at various times after TBI, ranging from initial injury (minutes to hrs) to delayed recovery (weeks). Total RNA is prepared and analyzed on the Affymetrix Mouse Whole Genome array. Perturbations are defined in vascular gene expression using GO and KEGG pathway analyses. Then the vasculome is mapped onto validated protein-protein interaction networks. A comprehensive database is generated and annotated, and all data will be open and freely accessible for the research community. In this annual report, we describe our progress for the first year of our research, i.e. completion of all *in vivo* TBI mouse models, measurement of neurological deficits in all TBI mice, and the isolation of TBI microvessels and mRNA extraction.

BODY

Original Objectives and Deliverables:

Our project had three objectives with three matching deliverables. First, we would execute the two mouse models of TBI – open-cranium impact and closed-cranium impact. The first deliverable (month 1-12) would be a full assessment of neurologic deficits on standard behavioral tests post-TBI. Second, we would extract and characterize microvessel mRNA from ipsilateral and contralateral TBI brains. The second deliverable (month 8-18) would be a full examination of mRNA to ensure quality and microvessel purity. Third, we would then run all extracted microvessel mRNA post-TBI on Affymetrix gene arrays. The third deliverable (month 15-24) would be the production of an annotated database of the TBI vasculome with GO, KEGG and PPI networks that can be compared with existing plasma protein databases.

Progress and Findings:

We successfully met all expected milestones thus far. For this first year annual report, we will describe our progress for Objective 1 and part of Objective 2. Male C57Bl6 mice were placed into a stereotactic frame within a standard computer-controlled pneumatic controlled cortical impact device (**Figure 1**). Mice were then subjected to either open-cranium or closed-cranium cortical impact TBI procedure. Open-cranium cortical impact TBI produced grossly-observable damage on the cortical brain surface of all mice, whereas closed-cranium cortical impact resulted in grossly normal-looking brains (**Figure 2**).

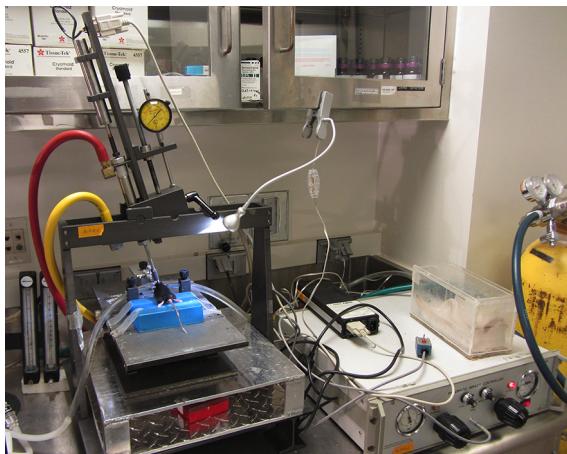


Figure 1



Figure 2

Over the course of the first few hrs to 21 days post-TBI, mice were evaluated with a battery of standardized tests, including the neurological severity score (NSS), the corner test, and foot-faults assessed with a wire-grip test. After open-cranium TBI, all mice developed severe neurological deficits that tended to be worst at 1-3 days post-injury, showed variable degrees of spontaneous recovery over time, but demonstrated remnant deficits even at the end of the observation period at 21 days (**Figure 3, see next page**). In contrast, mice subjected to closed-cranium TBI did not show severe deficits. Slight and variable deficits in NSS and foot-fault-wire-grip tests were recorded at 3 hrs post-injury, and all these rapidly renormalized so that by 21 days, mice appeared normal (**Figure 4, see next page**).

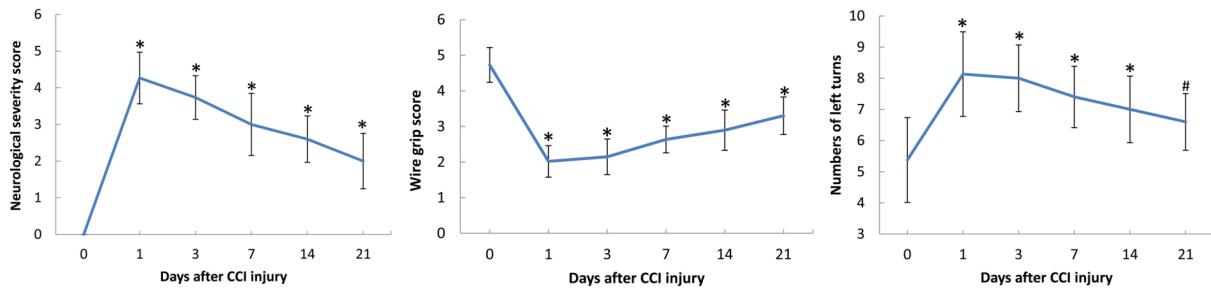


Figure 3

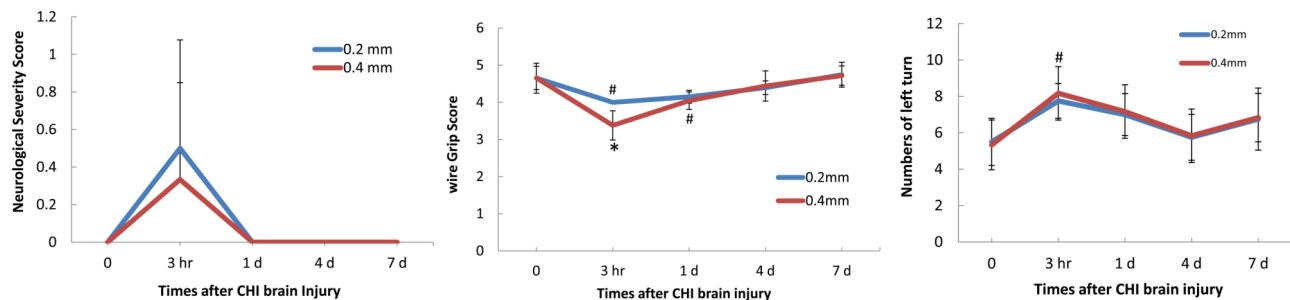


Figure 4

At each time point post-TBI, mice were killed and brains removed and microvessels were successfully purified from ipsilateral and contralateral hemispheres post-TBI in all mice. Based on these purified microvessels, mRNA was extracted using standard techniques.

In order to map the TBI vasculome, it is essential that two key quality controls steps are fulfilled. First, it is critical to demonstrate that mRNA quality is high enough for sufficient signal-to-noise to be obtained when samples are run on the full gene arrays. To accomplish this first step, mRNA quality in all samples was rigorously assessed with the Bioanalyzer method. We are pleased to report that mRNA quality passed this first quality-control step. Examples of Bioanalyzer runs are shown in **Figure 5**.

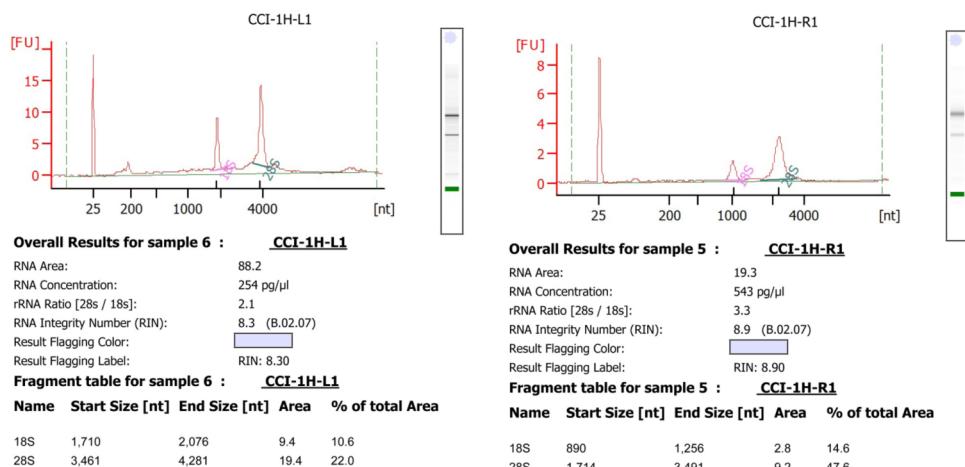


Figure 5

A second key step before we can use the full gene arrays to map the TBI vasculome is to ensure that our samples are truly pure microvessel fragments and not contaminated by neuronal or glial components. In order to do this, we ran independent RT-PCR assays on all samples and compared the mRNA expression levels of representative endothelial genes versus neuronal and glial genes. Endothelial genes were selected as *cdh5*, *enos*, *pecam1*. Neuronal genes were selected as *neurogranin* and *map2*. Astrocyte genes were selected as *aqp4* and *gfap*. Our RT-PCR data confirmed that our vasculome samples were indeed not contaminated (**Figure 6**).

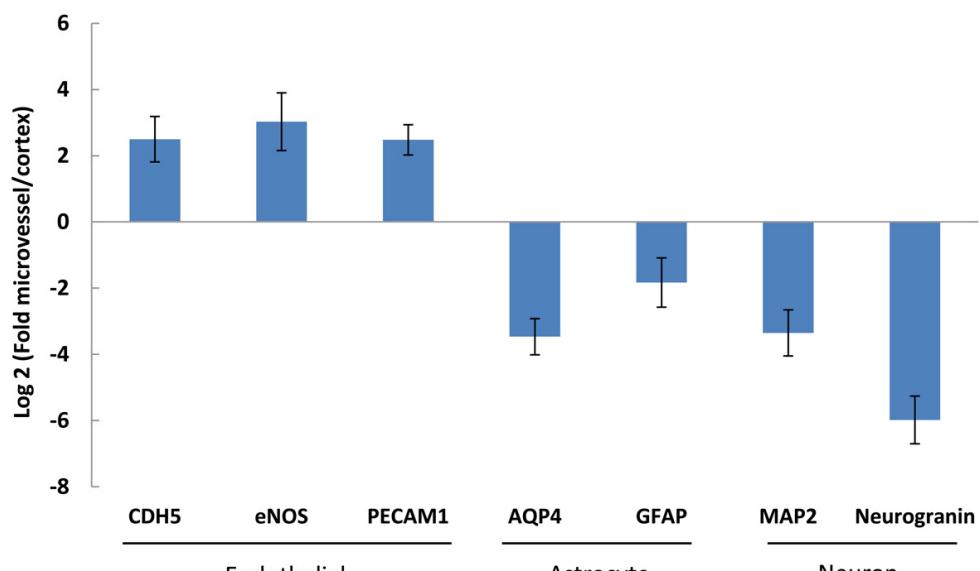


Figure 6

Taken together, we are pleased to report that we successfully met the milestones and produced the deliverables as expected for this first year annual report, i.e. we executed open-cranium impact and closed-cranium impact mouse models of TBI and collected the full dataset of neurologic deficits post-TBI (first deliverable, month 1-12), and we confirmed mRNA quality and obtained initial checks of microvessel purity for our TBI vasculome samples (second deliverable, month 8-18).

KEY RESEARCH ACCOMPLISHMENTS

- Compared the temporal evolution of neurobehavioral outcomes in open-cranium versus closed-cranium cortical impact in male mice.
- Showed that open-cranium cortical impact in mice produced significantly more severe sensorimotor and cognitive deficits compared to closed-cranium cortical impact over 21 days post-TBI.
- Isolated microvessels and extracted mRNA from TBI brains.
- Showed that high quality mRNA could be isolated in microvessels extracted from “morphologically-undamaged” ipsilateral brain tissue post-TBI, thus validating the feasibility of profiling the TBI vasculome.
- Obtained initial data confirming purity of TBI vasculome samples.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

- Guo S, Zhou Y, Xing C, Lok J, Som AT, Ning M, Ji X, Lo EH: The vasculome of the mouse brain. PLoS One. 2012; 7:e52665. PubMed PMID: 23285140; PubMed Central PMCID: PMC3527566.
Note: this performance of experiments for this paper was completed before the start of the present USA MRAA funding. However, the concepts proposed herein provide the direct basis for our current TBI vasculome project.
- 8 Feb 2013: Thomas Willis Award Lecture, International Stroke Conference
“Causation and collaboration for stroke research”
Note: this lecture was more focused on stroke rather than TBI. However, the concept of the vasculome was presented and discussed.
- 26 Feb 2013: Invited lecture, Scientific Advisory Symposium, Biomedical Research and Integrative Neuroscience Center University of New Mexico
“Mechanisms and challenges for translational neuroscience”
Note: this presentation focused on the challenges of translation. The vasculome was presented as a model system whereby cellular mechanisms may lead to clinically relevant outcomes and endpoints.
- 4 April 2013: Neuroscience Grand Rounds, University of Louisville, Louisville, KY
“Neurovascular mechanisms and challenges for translational research”
Note: Louisville is considered one of the leading TBI research and clinical centers in the world. The presentation of the vasculome concept was well received.
- 27 April 2013: Plenary Session: Traumatic brain injury – journey to recovery. 20th Annual Meeting, American Society for Neural Therapy and Repair
“Translational challenges for injury and repair: relevance to TBI”
Note: The 2013 meeting of the ASNTR was focused on TBI. This presentation was given in a special session on military TBI, along with leaders in the field such as Dr. Ronald Hayes (University of Florida), Lt. Col. Christine Stahl (McDill Air Force Base), and Dr. Steven Scott (Chief of Rehab, VA Hospital, Tampa). The concept of the TBI vasculome was presented and well received.
- 1 Oct 2013: Special Session: Frontiers in translation neuroscience. 11th Annual Meeting, NeuroCritical Care Society
“Neurovascular unit, MMPs and stem cells”
Note: The NeuroCritical Care Society is the leading society of physicians responsible for taking care of TBI patients acutely. This presentation emphasized the importance of the TBI vasculome concept.

patents and licenses applied for and/or issued: none

degrees obtained that are supported by this award: none

development of cell lines, tissue, or serum repositories: none

informatics such as databases and animal models: not yet

funding applied for based on work supported by this award: none

employment or research opportunities applied for and/or received based on experience/training supported by this award: none

CONCLUSIONS

As hypothesized, we were able extract viable high-quality mRNA from the TBI vasculome in mice. And as hypothesized, closed-cranium TBI resulted in highly subtle and inconsistent neurological deficits compared to the more severe standard open-cranium TBI models. These two models may now give us an opportunity to model the highly challenging, wide-spectrum, subtle and highly variable responses that may be observed in our military personnel who suffer from mild-to-moderate TBI.

This project remains important because it examines, for the first time, the novel concept that microvessels in brain are perturbed during mild-to-moderate TBI, even in “normal-looking” ipsilateral brain tissue. Why is this significant? There may be two potential reasons.

First, if the microvessels are indeed perturbed, even in the absence of clear and consistent neurological deficits and outright morphological damage to the brain parenchyma, they may release signals into circulating blood. This was our original hope. If we can map this TBI vasculome, this may provide a rich database that can be annotated and mined for potential biomarkers.

Second, besides releasing signals into blood, the TBI vasculome may also be a mechanistically critical source of abnormal signals into the brain itself. Hence, even in the absence of clear and consistent neurological deficits and outright morphological damage to the brain parenchyma, TBI microvessels may release factors that perturb neuronal and glial function.

Biomarkers are important, no question. But what this project has made us really excited about now, is the possibility of using the TBI vasculome to define novel mechanisms to explain why mild-to-moderate TBI can induce highly subtle neurological perturbations in the absence of clear and consistent neurological deficits and outright brain tissue damage. Although not part of this project, our lab has obtained pilot data using internal institutional funding (Massachusetts General Hospital) to show that perturbed cerebral endothelial cells may release microparticles that can activate microglia into potentially dangerous phenotypes. These activated microglia are very subtle and do not exhibit typical M1 characteristics. Beyond biomarkers per se, this aspect of the TBI vasculome may reveal new therapeutic targets.

REFERENCES: none.

APPENDIX: none.